Grape seed and linseed, alone and in combination, enhance unsaturated fatty acids in the milk of Sarda dairy sheep

F. Correddu, G. Gaspa, G. Pulina, A. Nudda,

Grape seed and linseed, alone and in combination, enhance unsaturated fatty acids in the milk of Sarda dairy sheep,

Journal of Dairy Science, Volume 99 Issue 3,2016,1725-1735,

https://doi.org/10.3168/jds.2015-10108.

Grape seed and linseed, alone and in combination, enhance the unsaturated fatty acids in the milk of Sarda dairy sheep

***Interpretive Summary*. Grape seed and linseed, alone and in combination, enhance the unsaturated fatty acids in the milk of Sarda dairy sheep. Correddu et al.** Grape seed is a winery by-product which contains a considerable amount of polyphenols and oils. Its use in ruminant nutrition could represent an alternative for their problematic management and disposal, and could be useful to increase the concentration of beneficial fatty acids in sheep milk. The aim of this study was to investigate the effect of dietary grape seed, alone or in combination with linseed (rich in polyunsaturated fatty acids), on milk fatty acid composition in lactating dairy ewes. Grape seed and linseed improve sheep milk quality due to a summative effect on fatty acids profile.

GRAPE SEED AND LINSEED FED TO DAIRY EWES

Grape seed and linseed, alone and in combination, enhance the unsaturated fatty acids in the milk of Sarda dairy sheep

F. Correddu, G. Gaspa, G. Pulina, and A. Nudda[1]

Dipartimento di Agraria, Sezione di Scienze Zootecniche, University of Sassari, Viale Italia 39, 07100 Sassari, Italy

[1]Corresponding author: Anna Nudda, Viale Italia, 39, 07100 Sassari, Italy, Tel.: +39 079229390, Fax: +39 079229302; e-mail address: anudda@uniss.it

ABSTRACT

This study evaluated the effect of the dietary inclusion of grape seed and linseed, alone or in combination, on sheep milk fatty acids (FA) profile using twenty-four Sarda dairy ewes allocated to four isoproductive groups. Groups were randomly assigned to four dietary treatments consisting of a control diet (CON), a diet including 300 g/d per head of grape seed (GS), a diet including 220 g/d per head of extruded linseed (LIN), and a diet including a mix of 300 g/d per head of grape seed and 220

g/d per head of extruded linseed (MIX). The study lasted 10 wk, with two wk of adaptation period and 8 wk of experimental period. Milk FA composition was analyzed in milk samples collected in the last four wk of the trial. The milk concentration of saturated fatty acids (SFA) decreased and that of unsaturated, monounsaturated and polyunsaturated fatty acids (UFA, MUFA and PUFA, respectively) increased in GS, LIN and MIX groups compared with CON. The MIX group showed the lowest values of SFA and the highest of UFA, MUFA and PUFA. Milk from ewes fed linseed (LIN and MIX) showed an enrichment of vaccenic acid (VA), oleic acid (OA), α-linolenic acid (LNA) and *cis*-9,*trans*-11 CLA compared with milk from the CON group. The GS group showed a greater content of milk oleic acid (OA) and linoleic acid (LA) and tended to show a greater content of VA and *cis*-9,*trans*-11 CLA than the CON group. The inclusion of grape seed and linseed, alone and in combination, decreased the milk concentration of *de novo* synthesized FA C10:0, C12:0, and C14:0, with the MIX group showing the lowest values. In conclusion, grape seed and linseed could be useful to increase the concentration of FA with potential health benefits, especially when these ingredients are included in combination in the diet.

Key words: sheep milk, beneficial fatty acids, grape seed, extruded linseed, by-product, multivariate analysis

INTRODUCTION

Growing interest in the nutraceutical properties of food has directed the attention of researchers to the improve the quality of dairy products. PUFA, such as PUFA *n*-3, are recognized to be beneficial to human health, by reducing serum triglycerides and low-density lipoprotein cholesterol (Simopoulos, 1991). Ovine milk is a major source of CLA, such as *cis*-9,*trans*-11 CLA (rumenic acid, RA), which has several effects, such as antiatherosclerotic, anticancer, antidiabetic and anti-inflammatory activity (Bhattacharya et al., 2006).

Diet is the most important factor influencing the milk FA composition in dairy ewes. In order to increase the concentration of nutritional FA in milk, sources of unsaturated plant lipids, such as linseed, soybeans, safflower and sunflower can be included in the diet (Nudda et al., 2014). In particular, linseed supplementation resulted in a high concentration of α-linolenic acid (LNA), CLA and vaccenic acid (C18:1 *trans*-11, VA), in milk of sheep, cows and goats (Zhang et al., 2006; Caroprese et al., 2010; Nudda et al., 2013a). Manipulation of ruminal biohydrogenation processes also may influence the milk FA composition. As demonstrated by in vitro and in vivo studies, dietary polyphenols can affect the growth and activity of some strains of bacteria involved in the biohydrogenation pathway of FA, leading to a shift in the ruminal microbial population (Vasta et al., 2009a, 2010). In particular, it has been evidenced that polyphenols can inhibit the proliferation and activity of *Butyrivibrio proteoclasticus*, involved in the last step of biohydrogenation of PUFA, which consists of the enzymatic reduction of VA to stearic acid (C18:0, **SA**) (Vasta et al., 2010; Buccioni et al., 2015). The consequent ruminal accumulation of PUFA and their biohydrogenation intermediates (Vasta et al., 2009b; Khiaosa-Ard et al., 2009) could enhance the extent of rumen escape of these FA and, consequently, could increase their concentration in milk, as demonstrated in studies on dairy cows and ewes (Moate et al., 2014; Buccioni et al., 2015).

Grape seed is a by-product derived from the winery and distillery industries which contains a high amount of polyphenols, mainly proanthocyanidins (Schieber et al., 2001). Therefore, the use of grape

seed in ruminant nutrition could be useful to modulate ruminal biohydrogenation of PUFA and could be an alternative for the expensive management and disposal of this by-product. The inclusion of grape residue in the diet of sheep increased rumen accumulation of VA (Correddu et al., 2015); in cows reduced methane emission, improved milk quality, by enhancing milk FA profile (Moate et al., 2014), and increasing antioxidant activity (Santos et al., 2014). Grape seed is also a good source of linoleic acid (C18:2 *n*-6, LA) which could positively affect the milk FA composition in dairy sheep.

Principal component analysis (PCA) and hierarchical cluster analysis (HCA) could be helpful methods to simplify the analysis of complex datasets composed of several variables, as the case of FA profile. In the last decades the use of multivariate analysis has become a popular approach to discriminate the effects of dietary treatments throughout the FA composition in meat (Coltro et al., 2005) and milk (Kadegowda et al., 2008) fat.

We hypothesized that dietary grape seed could enhance the effectiveness of linseed in increasing the concentration of polyunsaturated fatty acids in sheep milk. Therefore, the main objective of this work was to investigate the effect of the inclusion of grape seed in the diet of lactating ewes, alone or associated with linseed, on milk FA profile. Moreover, the multivariate analysis was used to test the hypothesis that data of milk FA could be useful tool to discriminate between groups of ewes fed diets with a different FA profile.

MATERIAL AND METHODS

The experiment was conducted in a dairy sheep farm located in the north-west of Sardinia from February to April 2013. The sheep management and the chemical analysis of feeds have been previously reported in detail by Correddu et al. (2015). Briefly, 24 Sarda dairy ewes were selected to form four groups balanced for milk production (1.75 ± 0.02 kg/d per head, mean ± SD), body weight (BW 43.2 ± 0.7 kg, mean ± SD), DIM and number of lactation (2-3 lactations). Each group was allocated to one of the following dietary treatments: control diet (CON), a diet containing 300 g/d per head of grape seed (GS), a diet containing 220 g/d per head of extruded linseed (LIN) and a diet

containing both 300 g/d of grape seed and 220 g/d of linseed per head (MIX). The ingredients, the chemical composition and the fatty acid profile of the experimental diets are reported in Table 1. All animals were fed a common ration, which included a commercial concentrate, beet pulp, mixed hay and dehydrated alfalfa hay, and a mixed meal, which included corn, soybean, pea, grape seed and linseed at varying amounts depending on the dietary treatment. The quantity of peas, soybeans and corn was calculated in order to make isoproteic diets and to supply the same level of metabolizable energy to each group. Linseeds were offered at the dose of 220 g/d per head in order to provide 70 g/d per head of fat. Grape seeds were offered at the dose of 300 g/d per head to provide approximately 1 g/d per head of total grape seed polyphenols, considering that the total phenolic content of grape seed was 333.3 ± 10.1 mg gallic acid equivalent (GAE)/100 g of dry matter (DM; mean ± S.E.).

Milk Samples

Individual morning milk samples were collected weekly and stored at −20°C until analysis. Milk samples collected in the last four wk of the trial were used to analyze the milk FA composition.

Fatty Acid Composition of Milk

Milk fat extraction and FAME preparation were performed as described by Nudda et al. (2005). The FAME were analyzed using a Turbo 3400 CX gas chromatograph (Varian Inc., Palo Alto, CA), equipped with a flame ionization detector (FID), an automatic injector 8200 CX (Varian Inc.) and a capillary column (CP-select CB for FAME; 100 m x 0.32 mm i.d., 0.25 μm film thickness, Varian Inc.). The temperature program was as follows: 75°C for 1 min, increased at 5°C/min to 148°C and at 8°C/min to 165°C, held for 35 min, then increased at 5.5°C/min to 210 °C and, finally, at 3°C/min to 230°C and held for 14 min. Helium (1 mL/min flow rate) was used as carrier gas with a pressure of 255.10 kPa. Split ratio was 1:100. Injector temperature was set at 225°C and detector temperature was set at 285°C. The FAME peaks were routinely identified by comparing their retention times with those of known standards and with published studies, as detailed in Nudda et al. (2005).Varian Star 3.4.1 software was used to compute the retention time and area of each individual FAME.

FA were reported as g/100 g of total FAME and groups of FA were calculated as follows: SFA, sum of the individual saturated fatty acids; unsaturated fatty acids (**UFA)**, sum of the individual unsaturated fatty acids; MUFA, sum of the individual monounsaturated fatty acids; PUFA, sum of the individual polyunsaturated fatty acids; trans fatty acids (**TFA**) sum of individual trans fatty acids, branched-chain fatty acids (**BCFA**), sum of individual branched-chain fatty acids; odd- and branched-chain fatty acids (**OBCFA**), sum of individual odd- and branched-chain fatty acids; short-chain fatty acids (**SCFA**), sum of the individual fatty acids from C4:0 to C10:0; medium-chain fatty acids (**MCFA**), sum of the individual fatty acids from C11:0 to C17:0; long-chain fatty acids (**LCFA**), sum of the individual fatty acids from C18:0 to C22:6 (DHA); PUFA *n*-3, sum of individual *n*-3 fatty acids; PUFA *n*-6, sum of individual *n*-6 fatty acids; CLA, sum of individual conjugated linoleic acids; Total C18:1, sum of individual C18:1 isomers; Total C18:2, sum of individual C18:2 isomers, Total C18:1-*cis*, sum of individual C18:1-cis isomers; Total C18:1-*trans*, sum of individual C18:1 trans isomers.

The nutritional properties of milk fat were estimated by the n-6 to n-3 ratio and three following indices: the atherogenic index (**AI**) and trombogenic index (**TI**) were calculated according to Ulbricht and Southgate (1991) except for the substitution of C18:0 with C12:0, as suggested by Nudda et al. (2013b): AI = [12:0 + (4 × 14:0) + 16:0]/[(PUFA) + (MUFA)], and TI = (14:0 + 16:0)/[(0.5 × MUFA) + (0.5 × *n*-6) + (3 × *n*-3) + (*n*-3:*n*-6)]; the hypocholesterolemic to hypercholesterolemic ratio (**h:H**) was calculated according to Fernández et al. (2007) as follows: h:H = [(sum of 18:1*cis*-9, 18:1*cis*-11, 18:2 *n*-6, 18:3 *n*-6,18:3 *n*-3, 20:3 *n*-6, 20:4 *n*-6, 20:5 *n*-3, 22:4 *n*-6, 22:5 *n*-3 and 22:6 *n*-3)/(14:0 + 16:0)].

To study the effect of the different diets on the capacity of desaturating SFA to Δ^9- UFA, the Δ^9-desaturase indices (**DI**) were calculated according to Schennink et al. (2008) as follows:

C10 index = [C10:1/(C10:0 + C10:1)] × 100;

C14 index = [C14:1 *cis*-9/(C14:0 + C14:1 *cis*-9)] × 100;

C16 index = [C16:1 *cis*-9/(C16:0 + C16:1 *cis*-9)] × 100;

C18 index = [C18:1 *cis*-9/(C18:0 + C18:1 *cis*-9)] × 100;

CLA index = [CLA *cis*-9,*trans*-11/(C18:1 *trans*-11 + CLA *cis*-9,*trans*-11)] × 100;

Total index = [(C10:1 + C14:1 *cis*-9 + C16:1 *cis*-9 + C18:1 *cis*-9 + CLA *cis*-9,*trans*-11)/(C10:0 + C14:0 + C16:0 + C18:0 + C18:1 *trans*-11+ C10:1 + C14:1 *cis*-9 + C16:1 *cis*-9 + C18:1 *cis*-9 + CLA *cis*-9,*trans*-11)] × 100.

Statistical Analysis

Milk FA data were analyzed by the PROC MIXED procedure of SAS version 9.2 (SAS Institute Inc., Cary, NC). The model included the fixed effect of diet (D; 4 levels), sampling date (S; 4 levels) and the diet × sampling date interaction (D × S); moreover, to account for individual variability, the random effect of animal was nested within each treatment. The significance of group mean differences was assessed using Tukey Honestly Significant Difference (HSD; $P < 0.05$).

A multivariate approach was also adopted to better clarify the effect of the four dietary treatments on the milk FA composition, using a dataset obtained from the average values of four sampling dates per animal. A total of 21 variables were analyzed (17 milk fatty acid groups and 4 nutritional indices) using hierarchical cluster analysis (HCA) and principal component analysis (PCA). HCA was performed on the milk FA profile using the Euclidean distances and the average linkage method. A dendrogram was used to visualize the clustering of the experimental units. Furthermore, the correlation matrix of milk fatty profiles was decomposed by the analysis of principal components (**PC**) as follows:

$$PC_j = \alpha_{1j}y_1 + \ldots + \alpha_{ij}y_i + \ldots + \alpha_{(n-1)j}y_{(n-1)} + \ldots + \alpha_{nj}y_n,$$

where n represents the number of variables (21), PC_j represents the generic *j*-th linear combination of the observed variables (scores) and α_{ij} the *i*-th coefficients of the eigenvector (loading) of correlation matrix, corresponding to the generic *j*-th eigenvalue (i.e., the variance explained by the *j*-th PC). The process of extraction was stopped when the variance explained by eigenvalues accounted for at least

80% of the total variance. Individual PC scores were then used in a one-way ANOVA including the fixed effect of treatments.

RESULTS AND DISCUSSION

Milk Fatty Acid Profile

The FA composition of milk collected from the ewes of the four experimental treatments is reported in Table 2. The concentration of C4:0 increased in milk of ewes fed grape seed in combination with linseed (MIX) compared with CON ($P < 0.05$). The mean values found for this FA (2.8% of FA) appear to be low when compared with those of other studies, which ranged from 3.1 to 4.6% (Gómez-Cortés et al., 2009; Buccioni et al., 2015). This difference could be the consequence of the volatilization of C4:0 during the extraction and methylation processes used in our analyses. The inclusion of linseed, alone or in combination with grape seed, reduced the concentration of FA from C6:0 to C9:0 ($P < 0.05$). A large part of the FA from C10:1 to C17:1 *cis*-9 decreased in the GS, LIN and MIX groups compared with CON ($P < 0.05$), except for *anteiso* C13:0, *iso* C14:0, C16:1 *trans*-6 + *trans*-7 and C16:1 *cis*-7, which did not differ ($P > 0.05$), and C16:1 *trans*-8, which increased in MIX, C16:1 *trans*-9, which increased in LIN and MIX, and C16:1 *cis*-10, which increased in GS, LIN and MIX, compared with CON ($P < 0.05$). These changes resulted in the reduction in SCFA and MCFA concentration in the milk of ewes fed GS, LIN and MIX, in decreasing order, compared with CON ($P < 0.05$). The LCFA concentration was higher in the treated groups than in the CON group ($P < 0.05$), in the following decreasing order: MIX, LIN and GS.

The concentration of total SFA decreased and that of UFA, MUFA and PUFA increased in all groups compared with CON ($P < 0.05$), with the lowest SFA and the highest UFA, MUFA and PUFA values being found in the MIX group ($P < 0.05$). The increase in UFA and PUFA, due to the dietary inclusion of grape seed, linseed or both, resulted in a higher UFA to SFA and PUFA to SFA ratios in all treated groups compared with CON ($P < 0.05$). The extent of increase of these ratios, especially

193 those of PUFA:SFA in the milk from the MIX group compared with CON (+ 187.5%) are very
194 interesting, considering that it has been evidenced that replacing dietary SFA with PUFA is likely to
195 reduce the occurrence of coronary heart disease (Mozaffarian et al., 2010).
196 The content of C18:0 increased in GS, LIN and MIX compared with CON ($P < 0.05$). The
197 concentration of most of the C18:1, C18:2 and CLA isomers increased in the milk of sheep fed linseed
198 (LIN and MIX) compared with that of CON ($P < 0.05$), in accordance with other studies on cows
199 (Caroprese et al., 2010; Ferlay et al., 2013), goats (Nudda et al., 2006; 2013a) and ewes (Mughetti et
200 al., 2012) fed linseed. These results were likely due to the fact that linseed is a rich source of C18:3
201 FA (> 55% of FA) and a moderate source of C18:1 and C18:2 (the sum is approximatly 33% of FA).
202 The presence of high concentrations of C18:1 isomers in the LIN and MIX groups can be partly
203 explained by the biohydrogenation of C18:2 and C18:3 FA in the rumen and of the desaturation of
204 SA in the mammary gland (Kennelly, 1996). The concentration of C18:1 *trans*-11 (vaccenic acid,
205 **VA**) increased ($P < 0.05$) in LIN and MIX compared with CON. This is consistent with the high
206 amount of linolenic acid (C18:3 *n*-3, **LNA**) supplied by linseed, considering that this FA is a precursor
207 of VA produced by the ruminal metabolism, and is in accordance with the experiments of Nudda et
208 al. (2006, 2013a) and Mughetti et al. (2012), in which dietary linseed increased the levels of VA in
209 milk of dairy goats and sheep. VA is the precursor of the CLA *cis*-9,*trans*-11 formed in the mammary
210 gland by the Δ^9-desaturase (Griinari and Bauman 1999). In fact, in our study the concentration of
211 CLA *cis*-9,*trans*-11 in the milk of the groups fed linseed (LIN and MIX) was higher ($P < 0.05$) than
212 in those of the CON group. The level of CLA *cis*-9,*trans*-11 concentration in the milk from the LIN
213 group (2.16% of FAME) was comparable to that reported for sheep grazing high-quality pasture
214 (2.20% of FAME, Nudda et al., 2005) or fed a similar dose of linseed (2.33% of FAME, Gómez-
215 Cortés et al., 2009). Interestingly, the concentration of CLA *cis*-9,*trans*-11 (1.73% of FAME) in milk
216 from sheep fed grape seed (GS), which was numerically but not significantly higher than CON, was
217 similar to that reported for sheep fed high amounts of linseed oil (about 40 g/d; Zhang et al., 2006) or

fish oil (30 g/d; Mozzon et al., 2002). In the present work, milk from ewes fed grape seed and linseed in combination (MIX) had a high concentration of CLA *cis*-9,*trans*-11 (3.0% of FAME). As reported in the review by Nudda et al. (2014), concentrations of CLA *cis*-9,*trans*-11 higher than 3% of fat have been previously reached by using a very high dose of soybean oil (140 g/d) associated with a high-concentrate diet. Dietary linseed also increased ($P < 0.05$) the concentration of LNA in milk from LIN (1.87% of FAME) and MIX (1.42% of FAME) compared with CON and GS. The extent of enrichment of LNA was consistent with previous studies where linseed was included in the diet of sheep (Mele et al., 2007; Gómez-Cortés et al., 2009)

The presence of a moderate concentration of polyphenols in the diet increased the level of beneficial FA, mainly LNA, in milk from ewes (Cabiddu et al., 2009) and cows (Dschaak et al., 2011). This effect has been explained by the capacity of polyphenols to inhibit the activity of some strains of ruminal bacteria involved in the biohydrogenation of UFA (Cabiddu et al., 2009; Vasta et al., 2009a; Minieri et al., 2014). In this work, the inclusion of grape seed, alone or in combination with linseed, increased the concentration of PUFA compared with the CON group ($P < 0.001$). However, this increase was likely due to the high amount of LA in grape seeds (about 75% of FA), considering that GS and MIX also increased LA and, consequently, PUFA *n*-6 in milk compared with CON and LIN ($P < 0.05$). This is in agreement with the findings of Moate et al. (2014) and Santos et al. (2014), who showed increased levels of LA in milk of dairy cows fed grape residue.

The concentration of PUFA *n*-3, which was the lowest in CON and GS, was higher in milk from sheep fed linseed alone than in combination with grape seed ($P < 0.001$). This is likely due to the lack of effect of grape seed in reducing the extent of biohydrogenation of LNA, as suggested by the decreased level of LNA in milk of MIX compared with LIN ($P < 0.05$) and the similarly low levels of LNA in CON and GS. The low level of polyphenols in the grape seed used in the present work, compared with those of other studies, could explain the lack of effect of this ingredient in increasing the concentration of LNA in milk of GS compared with CON, but does not explain the reduction in

243 LNA in milk of MIX compared with that of LIN group. Therefore, considering that grape seed
244 contains other compounds that could have affected the biohydrogenation of UFA, we hypothesize
245 that the presence of grape seed might have increased, to some extent, the biohydrogenation of dietary
246 PUFA, as suggested by the higher concentration of VA in MIX than in LIN and in GS than in CON,
247 even though these differences were not statistically significant ($P < 0.10$). Our results are in
248 accordance with the study of Moate et al. (2014), in which the milk concentration of LNA did not
249 increase in lactating cows fed grape marc. The pattern of the concentration of PUFA *n*-3 mirrored
250 that of LNA, with CON and GS showing lower PUFA *n*-3 values than LIN, and MIX being
251 intermediate ($P < 0.05$).

252 The inclusion of grape seed and linseed in the diet of sheep, especially when offered in
253 combination (MIX), increased the concentration of milk TFA compared with CON ($P < 0.05$). This
254 result mirrored the increase in most of the individual TFA in those groups compared with CON, likely
255 as a consequence of rumen biohydrogenation of PUFA, whose dietary concentration followed the
256 increasing order CON < GS < LIN < MIX (Table 1). This finding is in agreement with a previous
257 study showing an increased concentration of TFA in milk when extruded linseed was included as
258 source of PUFA in the diet of dairy cows (Livingstone et al., 2015). These results were influenced
259 the most by VA, which accounted for 34.21, 40.42, 43.59 and 46.78% of the total TFA in milk from
260 CON, GS, LIN and MIX, respectively.

261 The total concentration of OBCFA decreased in the milk of the GS, LIN and MIX groups
262 compared with CON, being the lowest in MIX and intermediate in GS and LIN ($P < 0.05$). OBCFA
263 are reported to be mainly derived from the ruminal microflora (Fievez et al., 2012). The decrease in
264 OBCFA in milk of LIN could be explained by the high amount of PUFA, particularly LNA, in linseed,
265 considering that PUFA are reported to be toxic for the growth of ruminal microorganisms (Maia et
266 al., 2007, 2010). Similarly, the high concentration LA in grape seed could explain the reduction in
267 OBCFA in milk from GS compared with CON. Moreover, according with several studies showing

268 the effect of polyphenols on the growth and activity of rumen microbial population (Vasta et al.,
269 2010; Buccioni et al., 2015), the grape seed polyphenols could have contributed to this reduction. The
270 high amount of PUFA, mainly LNA and LA, and the presence of polyphenols in the MIX diet could
271 be the reason for the lowest concentration of OBCFA found in the milk from sheep of this group, as
272 confirmed by the previously reported results of the analysis on rumen liquid FA profile of the ewes
273 of the dietary groups under comparison (Correddu et al., 2015).

274 The inclusion of grape seed and linseed, alone and in combination, decreased ($P < 0.05$) the milk
275 concentration of de novo synthesized FA C10:0, C12:0, and C14:0 compared with the CON group,
276 probably due to the increase in the amount of PUFA in the diet of sheep, in accordance with previews
277 studies in lactating sheep (Zhang et al., 2006), goats (Bernard et al., 2009) and cows (Chilliard et al.,
278 2007). In addition, the concentrations of C10:1, C14:1 *cis*-9, C16:1 *cis*-9 and C17:1 *cis*-9 were also
279 lower in milk of GS, LIN and MIX compared with CON ($P < 0.05$). As suggested by Bernard et al.
280 (2009), an increase in the amount of TFA and PUFA can reduce the activity of stearoyl Co-A
281 desaturase in the mammary gland and, consequently, the extent of Δ^9-desaturation of C10:0, C14:0,
282 C16:0 and C17:0. The analysis of the desaturase indices partly confirmed these results. In particular,
283 CON showed higher values of the C18 and CLA indices ($P < 0.05$) than MIX and the other two
284 groups being intermediate, whereas the C10, C14 and C16 indices were not significantly influenced
285 by the diets ($P > 0.05$). Although the concentration of C18:1 *cis*-9 and CLA *cis*-9,*trans*-11 increased
286 with the inclusion of grape seed and linseed, the DI related to these FA did not follow the same
287 pattern, suggesting that the increase in these FA was not related to an increasing activity of Δ^9-
288 desaturase but, more likely, to the increase in the concentration of their substrates C18:0 and C18:1
289 *trans*-11. The total DI increased in all groups compared with CON ($P < 0.05$), even if the individual
290 DI followed an opposite trend. This is in contrast with the positive correlation between all DI
291 (individual and total) observed by Schennink et al. (2008), and could be explained by differences
292 between these studies in the ratio between C18:1 *cis*-9 and C16:0, which are the most abundant FA

293 in milk. As pointed out by Schennink et al. (2008), the value of total DI mirrors mainly the ratio
294 C18:1 *cis*-9 to C16:0. The opposite trend between individual DI and total DI found in the present
295 work suggests that the total DI is not a reliable indicator of the desaturation activity of stearoyl Co-A
296 desaturase.

297 As shown in Figure 1, the dietary inclusion of grape seed and linseed was effective in reducing the
298 atherogenic and thrombogenic indices, and increasing the h:H ratio compared with CON ($P < 0.05$).
299 Our results are consistent with the fact that dietary sources of PUFA ameliorate cardiac risk factors
300 (Duda et al., 2009, Katare and Saxena, 2013), and with a previous study in which dietary extruded
301 linseed decreased the values of AI and TI and increased the h:H ratio in dairy goats (Nudda et al.,
302 2013a). Similar results were found in Lacaune ewes fed extruded linseed (Casamassima et al., 2014).
303 The effect of the dietary inclusion of grape seed on these indices was likely related to the large
304 decrease in C12:0, C14:0 and C16:0 and increase in MUFA. The values of TI were lower in LIN than
305 in GS ($P < 0.05$), suggesting that the inclusion of linseed is more effective in increasing the
306 concentration of beneficial FA than grape seed. Grape seed and linseed in combination (MIX) led to
307 lower values of the AI and TI indices, and a higher value of h:H than grape seed alone ($P < 0.05$).
308 Moreover, the reduction in AI and TI and the increase in h/H were numerically higher, although not
309 statistically different, in MIX than in LIN, suggesting a summative effect of linseed and grape seed.
310 The substantial improvement in milk FA due to the combined effect of grape seed and linseed is
311 evidenced by the 65.33 and 62.61% decrease in AI and TI, respectively, and by the 125% increase in
312 h:H in MIX compared with CON.

313 Most of the FA measured during the trial were influenced by sampling date, with FA of the same
314 class generally showing a similar pattern (data not shown). In particular, most of the SFA, SCFA and
315 MCFA showed a significant decrease ($P < 0.05$) in the second and third samplings compared with
316 the first and last samplings, whereas most of the UFA, MUFA, PUFA and LCFA showed an opposite
317 trend, with the second and third samplings showing greater values than the first and last samplings

($P < 0.05$). Although many of the FA were significantly influenced by the D × S interaction ($P < 0.05$), the few differences observed in the temporal pattern among dietary treatments (data not shown) was not relevant compared with the main effect of the diet on FA concentration.

Multivariate Analysis

The results of the PCA are shown in Table 3 and Figure 2. Two principal components were retained for subsequent analysis based on the proportion of variance explained by each PC. The first and second principal components accounted for about 90% of the total variability (78% and 12% for PC1 and PC2, respectively). Table 3 shows the eigenvalues and eigenvectors of the correlation matrix derived from groups of FA in milk. The PC1 was positively associated with the groups of FA characterized by long and unsaturated chains, whereas it was negatively associated with groups characterized by short- and medium-chain FA and saturated FA. The PC1 was also positively correlated with the sum of C18:1 and C18:2 isomers and, among C18:1, the *trans* isomers showed a greater correlation than the *cis* isomers. According to previous studies on dairy cows, the dietary supplementation with vegetable oils as source of PUFA increased the concentration of long-chain PUFA *n*-3 (Ferlay et al., 2013) or PUFA *n*-6 (Almeida et al., 2013), and decreased the concentration of short- and medium-chain FA in milk. Among PUFA, PC2 loadings were positively correlated with *n*-6 and *n*-6 to *n*-3 ratio, and negatively with *n*-3. Moreover, PC2 negatively discriminated the OBCFA and BCFA. PC1 showed high positive loadings for the h:H ratio and high negative loadings for the AI and TI indices. PC2, to a lesser extent, was positively correlated with the AI and TI and negatively with the h:H ratio.

The plot of the first two PC scores allowed the description of the relationship among animals based exclusively on the milk FA profile (Figure 2). Four clusters were identified according to the four dietary treatments, with the CON being the most isolated group and being mainly discriminated by PC1 (negative scores). PC2 scores discriminated GS (positive scores) from LIN (negative scores). We suppose that PC1 was positively associated with the dietary inclusion of PUFA, especially with

343 the PUFA intake (CON < GS < LIN < MIX, as previously reported in Correddu et al., 2015);
344 therefore, PC1 was named "PUFA intake". The PC2 could be related to the different sources of PUFA
345 (grape seed or linseed) and consequently, to the PUFA *n-6* to *n-3* ratio in the diets; thus, PC2 was
346 identified as the "*n-6* to *n-3* ratio". Similar results were reported in the work of Bernard et al. (2009),
347 who investigated the effects of sunflower and linseed oils, characterized by high LA and LNA
348 content, respectively, on goat milk fatty acid composition. In that study, the analysis of principal
349 component, used to clarify the relationship between the oil treatments, forages and milk production
350 and composition, showed that PC1 was related with the lipid supplementation, and PC2 was related
351 to the content of LA and LNA in the diets.
352 The results of the HCA performed in the present study are shown in Figure 3. The dendrogram
353 allowed to group the animals in four clusters, with 72.80% of similarity level. The animals of CON
354 formed a unique cluster, indicating that the chemical composition of milk from this group was
355 different from the milk composition of the other groups. Another unique cluster grouped the animals
356 of GS, indicating that the chemical composition of milk from sheep fed grape seed was different from
357 that of the CON and from those of the sheep fed linseed (LIN and MIX). The animals of the LIN and
358 MIX groups formed two clusters, except for a few cases of incorrect assignation: two animals of the
359 MIX treatment were assigned to the LIN group, and one animal of the LIN treatment was assigned to
360 the MIX group. This suggests that the chemical composition of the milk from sheep fed linseed (LIN
361 and MIX) was different from those of the CON and GS groups. The clustering of animals in the four
362 dietary treatments evidenced by the plot of principal components (Figure 2) and by the dendrogram
363 (Figure 3) was confirmed by the results of the statistical analysis of the relationship between dietary
364 groups and PC1 and PC2 reported in Table 4.
365
366 **CONCLUSIONS**

The dietary inclusion of grape seed or linseed, or both, improved the milk FA composition in Sarda dairy ewes and the multivariate approach allowed the detection of differences between dietary treatments based on the milk fatty acid profile. When grape seed was supplied alone, at 300 g/d per head, the milk content of SFA decreased and that of UFA and PUFA increased, mainly due to a high increase of LA, whereas the concentration of RA and VA tended to increase compared to the control group. The inclusion of 200 g/d per head of linseed alone in the diet of lactating ewes increased the concentration of potentially beneficial FA, such as oleic acid, linolenic acid, and CLA *cis*-9,*trans*-11. The inclusion of grape seed and linseed in combination resulted in a major increase of ratios UFA:SFA and PUFA:SFA and of the concentration of CLA *cis*-9,*trans*-11. In conclusion, the use of grape seed in sheep nutrition could be an alternative for the disposal of this by-product, and its combination with linseed could be a successful strategy to enhance PUFA in lactating Sarda ewes.

ACKNOWLEDGEMENTS

The authors thank Ana Helena Dias Francesconi, from the University of Sassari, for revising the manuscript, Antonio Fenu, Antonio Mazza, Roberto Rubattu and Gesumino Spanu, from the same University, for giving technical assistance, the Azienda Ledda for making the farm and animals available, and Giovanni Pinna from Cargill s.r.l. (Animal Nutrition Division) for providing the feed ingredients.

REFERENCES

Almeida, O. C., A. V. Pires, I. Susin, R. S. Gentil, C. Q. Mendes, M. A. A. Queiroz, E. M. Ferreira, and M. L. Eastridge. 2013. Milk fatty acids profile and arterial blood milk fat precursors concentration of dairy goats fed increasing doses of soybean oil. Small Rum. Res. 114:152−160.

390 Bernard, L., K. J. Shingfield, J. Rouel, A. Ferlay, and Y. Chilliard. 2009. Effect of plant oils in the
391 diet on performance and milk fatty acid composition in goats fed diets based on grass hay or
392 maize silage. Brit. J. Nutr. 101:213−224.
393 Bhattacharya, A., J. Banu, M. Rahman, J. Causey, and G. Fernandes. 2006. Biological effects of
394 conjugated linoleic acids in health and disease. J. Nutr. Biochem. 17:789–810.
395 Buccioni, A., M. Pauselli, C. Viti, S. Minieri, G. Pallara, V. Roscini S. Rapaccini, M. Trabalza
396 Marinucci, P. Lupi, G. Conte, and M. Mele. 2015. Milk fatty acid composition, rumen microbial
397 population, and animal performances in response to diets rich in linoleic acid supplemented with
398 chestnut or quebracho tannins in dairy ewes. J. Dairy Sci. 98:1145–1156.
399 Cabiddu, A., G. Molle, M. Decandia, S. Spada, M. Fiori, G. Piredda, and M. Addis. 2009. Responses
400 to condensed tannins of flowering sulla (*Hedysarum coronarium* L.) grazed by dairy sheep: Part
401 2: Effects on milk fatty acid profile. Livest. Sci. 123:230–240.
402 Caroprese, M., A. Marzano, R. Marino, G. Gliatta, A. Muscio, and A. Sevi. 2010. Flaxseed
403 supplementation improves fatty acid profile of cow milk. J. Dairy Sci. 93:2580–2588.
404 Casamassima, D., M. Nardoia, M. Palazzo, F. Vizzarri, A. G. D'Alessandro, and C. Corino. 2014.
405 Effect of dietary extruded linseed, verbascoside and vitamin E supplements on yield and quality
406 of milk in Lacaune ewes. J. Dairy Res. 81:485–493.
407 Chilliard, Y., F. Glasser, A. Ferlay, L. Bernard, J. Rouel, and M. Doreau. 2007. Diet, rumen
408 biohydrogenation and nutritional quality of cow and goat milk fat. Eur. J. Lipid Sci. Tech.
409 109:828−855.
410 Coltro, W. K., M. M. Ferreira, F. A. Macedo, C. C. Oliveira, J. V. Visentainer, N. E. Souza, and M.
411 Matsushita. 2005. Correlation of animal diet and fatty acid content in young goat meat by gas
412 chromatography and chemometrics. Meat Sci. 71:358−363.

413 Correddu, F., A. Nudda, G. Battacone, R. Boe, A. H. D. Francesconi, and G. Pulina. 2015. Effects of
414 grape seed supplementation, alone or associated with linseed, on ruminal metabolism in Sarda
415 dairy sheep. Anim. Feed Sci. and Technol. 199:61–72.
416 Dschaak, C. M., C. M. Williams, M. S. Holt, J.-S. Eun, A. J. Young, and B. R. Min. 2011. Effects of
417 supplementing condensed tannin extract on intake, digestion, ruminal fermentation, and milk
418 production of lactating dairy cows. J. Dairy Sci. 94:2508–2519.
419 Duda, M. K., K. M. O'Shea, and W. C. Stanley. 2009. ω-3 polyunsaturated fatty acid supplementation
420 for the treatment of heart failure: mechanisms and clinical potential. Cardiovasc. Res. 84:33–41.
421 Ferlay, A., M. Doreau, C. Martin, and Y. Chilliard. 2013. Effects of incremental amounts of extruded
422 linseed on the milk fatty acid composition of dairy cows receiving hay or corn silage. J. Dairy
423 Sci. 96:6577–6595.
424 Fernández, M., J. A. Ordóñez, I. Cambero, C. Santos, C. Pin, and L. de la Hoz. 2007. Fatty acid
425 compositions of selected varieties of Spanish dry ham related to their nutritional implications.
426 Food Chem. 101:107–112.
427 Fievez, V., E. Colman, J. M. Castro-Montoya, I. Stefanov, and B. Vlaeminck. 2012. Milk odd- and
428 branched-chain fatty acids as biomarkers of rumen function—An update. Anim. Feed Sci.
429 Technol. 172:51–65.
430 Gómez-Cortés, P., A. Bach, P. Luna, M. Juárez, and M. A. de la Fuente. 2009. Effects of extruded
431 linseed supplementation on n-3 fatty acids and conjugated linoleic acid in milk and cheese from
432 ewes. J. Dairy Sci. 92:4122–4134.
433 Griinari, J. M. and D. E. Bauman. 1999. Biosynthesis of conjugated linoleic acid and its incorporation
434 into meat and milk in ruminants. Pages 180–200 in Advances in Conjugated Linoleic Acid
435 Research, Vol. 1. M. P. Yurawecz, M. M. Mossoba, J. K. G Kramer, M. W. Pariza, and G. J.
436 Nelson, ed. AOCS Press, Champaign, IL.

437 Kadegowda, A. K., L. S. Piperova, and R. A. Erdman. 2008. Principal component and multivariate
438 analysis of milk long-chain fatty acid composition during diet-induced milk fat depression. J.
439 Dairy Sci. 91:749–759.
440 Katare, C., and S. Saxena. 2013. Amelioration of selected cardiac risk factors through
441 supplementation of diet with flaxseed and soya bean. Int. J. Nut. Pharmacol. Neurol. Dis. 3:352–
442 357.
443 Kennelly, J. J. 1996. The fatty acid composition of milk fat as influenced by feeding oilseeds. Anim.
444 Feed Sci. Technol. 60:137–152.
445 Khiaosa-Ard, R., S. F. Bryner, M. R. L. Scheeder, H. R. Wettstein, F. Leiber, M. Kreuzer, and C. R.
446 Soliva. 2009. Evidence for the inhibition of the terminal step of ruminal α-linolenic acid
447 biohydrogenation by condensed tannins. J. Dairy Sci. 92:177–188.
448 Livingstone, K. M., D. J. Humphries, P. Kirton, K. E. Kliem, D. I. Givens, and C. K. Reynolds. 2015.
449 Effects of forage type and extruded linseed supplementation on methane production and milk
450 fatty acid composition of lactating dairy cows. J. Dairy Sci. 98:4000–4011.
451 Maia, M. R. G., L. C. Chaudhary, C. S. Bestwick, A. J. Richardson, N. McKain, T. R. Larson, I. A.
452 Graham, and R. J. Wallace. 2010. Toxicity of unsaturated fatty acids to the biohydrogenating
453 ruminal bacterium, *Butyrivibrio fibrisolvens*. BMC Microbiol. 10:52.
454 Maia, M. R. G., L. C. Chaudhary, L. Figueres, and R. J. Wallace. 2007. Metabolism of
455 polyunsaturated fatty acids and their toxicity to the microflora of the rumen. Antonie. van
456 Leeuwenhoek. 91:303–314.
457 Mele, M., A. Serra, G. Conte, A. Pollicardo, M. del Viva, and P. Secchiari. 2007. Whole extruded
458 linseed in the diet of dairy ewes during early lactation: Effect on the fatty acid composition of
459 milk and cheese. Ital. J. Anim. Sci., 6:560–562.

460 Minieri, S., A. Buccioni, S. Rapaccini, A. Pezzati, D. Benvenuti, A. Serra, and M. Mele. 2014. Effect
461 of Quebracho tannin extract on soybean and linseed oil biohydrogenation by solid associated
462 bacteria: an *in vitro* study. Ital. J. Anim. Sci. 13:604–608.
463 Moate, P. J., S. R. O. Williams, V. A. Torok, M. C. Hannah, B. E. Ribaux, M. H. Tavendale, R. J.
464 Eckard, J. L. Jacobs, M. J. Auldist, and W. J. Wales. 2014. Grape marc reduces methane
465 emissions when fed to dairy cows. J. Dairy Sci. 97:5073–5087.
466 Mozaffarian, D., R. Micha, and S. Wallace. 2010. Effects on coronary heart disease of increasing
467 polyunsaturated fat in place of saturated fat: a systematic review and meta-analysis of
468 randomized controlled trials. PLoS Med. 7:e1000252.
469 Mozzon, M., N. G., Frega, B. Fronte, and M. Tocchini. 2002. Effect of dietary fish oil supplements
470 on levels of n-3 polyunsaturated fatty acids, *trans* acids and conjugated linoleic acid in ewe milk.
471 Food Technol. Biotech. 40:213–219.
472 Mughetti, L., F. Sinesio, G. Acuti, C. Antonini, E. Moneta, M. Peparaio, and M. Trabalza-Marinucci.
473 2012. Integration of extruded linseed into dairy sheep diets: Effects on milk composition and
474 quality and sensorial properties of Pecorino cheese. Anim. Feed Sci. Technol. 178:27–39.
475 Nudda, A., G. Battacone, A. S. Atzori, C. Dimauro, S. P. G. Rassu, P. Nicolussi, P. Bonelli, and G.
476 Pulina. 2013a. Effect of extruded linseed supplementation on blood metabolic profile and milk
477 performance of Saanen goats. Animal 7:1464–1471.
478 Nudda, A., G. Battacone, R. Boe, M. G. Manca, S. P. G. Rassu, and G. Pulina. 2013b. Influence of
479 outdoor and indoor rearing system of suckling lambs on fatty acid profile and lipid oxidation of
480 raw and cooked meat. Ital. J. Anim. Sci. 12:459–467.
481 Nudda, A., G. Battacone, M. G. Usai, S. Fancellu, and G. Pulina. 2006. Supplementation with
482 extruded linseed cake affects concentrations of conjugated linolenic acid and vaccenic acid in
483 goat milk. J. Dairy Sci. 89:277–282.

484 Nudda, A., G. Battacone, O. Boaventura Neto, A. Cannas, A. H. D. Francesconi, A. S. Atzori, and G.
485 Pulina. 2014. Feeding strategies to design the fatty acid profile of sheep milk and cheese. R. Bras.
486 Zootec. 43:445–456.
487 Nudda, A., M. A. McGuire, G. Battacone, and G. Pulina. 2005. Seasonal variation in conjugated
488 linoleic acid and vaccenic acid in milk fat of sheep and its transfer to cheese and ricotta. J. Dairy
489 Sci. 88:1311–1319.
490 Santos, N. W., G. T. D. Santos, D. C. Silva-Kazama, P. A. Grande, P. M. Pintro, F. E. de Marchi, C.
491 C. Jobim, and H. V. Petit. 2014. Production, composition and antioxidants in milk of dairy cows
492 fed diets containing soybean oil and grape residue silage. Livest. Sci. 159:37–45.
493 Schennink, A., J. M. L. Heck, H. Bovenhuis, M. H. P. W. Visker, H. J. F. van Valenberg, and J. A.
494 M. van Arendonk. 2008. Milk fatty acid unsaturation: genetic parameters and effects of stearoyl-
495 CoA desaturase (*SCD1*) and acyl CoA: diacylglycerol acyltransferase 1 (*DGAT1*). J. Dairy Sci.
496 91:2135–2143.
497 Schieber, A., F. C. Stintzing, and R. Carle. 2001. By-products of plant food processing as a source of
498 functional compounds – recent developments. Trends Food Sci. Tech. 12:401–413.
499 Simopoulos, A. P., 1991. Omega-3 fatty acids in health and disease and in growth and development.
500 Am. J. Clin. Nutr. 54: 438–463.
501 Ulbricht T. L. V. and D. A. T. Southgate. 1991. Coronary heart disease: seven dietary factors. Lancet,
502 338:985–992.
503 Vasta, V., H. P. S. Makkar, M. Mele, and A. Priolo. 2009a. Ruminal biohydrogenation as affected by
504 tannins *in vitro*. Br. J. Nutr. 102:82–92.
505 Vasta, V., M. Mele, A. Serra, M. Scerra, G. Luciano, M. Lanza, and A. Priolo. 2009b. Metabolic fate
506 of fatty acids involved in ruminal biohydrogenation in sheep fed concentrate or herbage with or
507 without tannins. J. Anim. Sci. 87:2674−2684.

508 Vasta, V., D. R. Yáñez-Ruiz, M. Mele, A. Serra, G. Luciano, M. Lanza, L. Biondi, and A. Priolo.
509 2010. Bacterial and protozoal communities and fatty acid profile in the rumen of sheep fed a diet
510 containing added tannins. Appl. Environ. Microbiol. 76:2549–2555.
511 Zhang, R. H., A. F. Mustafa, and X. Zhao, 2006. Effects of feeding oilseeds rich in linoleic and
512 linolenic fatty acids to lactating ewes on cheese yield and on fatty acid composition of milk and
513 cheese. Anim. Feed Sci. Technol. 127:220–233.
514

515 **Table 1.** Ingredients, chemical composition, dry matter intake and fatty acid profile of diets

	Diet[1]			
	CON	GS	LIN	MIX
Ingredients (kg/day per head, as fed)				
Mixed meal				
Corn	0.15	0.17	–	–
Soybean	0.12	0.24	0.04	0.16
Peas	0.25	0.09	0.15	0.02
Grape seed	–	0.30	–	0.30
Linseed	–	–	0.22	0.22
Beet pulp	0.40	0.40	0.40	0.40
Commercial concentrate	0.50	0.50	0.50	0.50
Dehydrated alfalfa hay	0.80	0.80	0.80	0.80
Mixed hay	0.20	0.20	0.20	0.20
Chemical composition, % of DM (unless otherwise noted)				
DM (%)	90.8	91.6	91.2	92.0
NDF	41.8	42.8	43.7	44.5
NFC	33.4	28.9	28.5	24.2
ADL	4.6	8.9	5.0	9.4
CP	18.0	17.9	17.9	17.9
Ash	7.8	7.4	8.1	7.6
EE	2.0	3.2	5.1	5.8
FA	1.8	2.3	3.7	4.5
ME supplied (Mcal/d)	4.95	4.94	4.97	4.97
Dry Matter intake (kg/d)	2.20	2.47	2.11	2.39
Major fatty acids (g/100 g of total FA)				
C16:0	18.98	14.88	11.99	11.50
C18:0	3.33	4.47	4.39	4.68
C18:1 *cis*-9	22.79	23.52	21.78	21.91
C18:2 *n*-6 (LA)	41.53	47.50	23.84	33.46
C18:3 *n*-3 (LNA)	8.25	5.04	34.45	24.93
SFA	24.24	20.88	17.75	17.42
MUFA	25.84	26.02	23.70	23.94
PUFA	49.92	53.11	58.55	58.64

516 [1]Diet: CON = control diet; GS = diet containing 300 g/d per head of grape seed; LIN = diet containing
517 220 g/d per head of linseed; MIX = diet containing 300 g/d of grape seed and 220 g/d of linseed per
518 head.
519

520 **Table 2.** Fatty acid profile of milk from sheep fed different experimental diets

Fatty acid (g/100 g of FAME)[3]	Diet[1]				SEM	*P*-value[2]		
	CON	GS	LIN	MIX		D	S	D × S
C4:0	2.58^{b}	2.86^{ab}	2.83^{ab}	2.96^{a}	0.034	*	***	*
C6:0	2.10^{a}	1.97^{ab}	1.65^{bc}	1.37^{c}	0.043	***	**	***
C8:0	2.20^{a}	1.86^{ab}	1.43^{bc}	1.03^{c}	0.058	***	***	**
C9:0	0.05^{a}	0.04^{b}	0.02^{c}	0.02^{c}	0.002	***	***	***
C10:0	8.98^{a}	6.33^{b}	4.63^{bc}	3.25^{c}	0.251	***	***	*
C10:1	0.35^{a}	0.25^{b}	0.18^{bc}	0.11^{c}	0.010	***	***	**
C11:0	0.10^{a}	0.05^{b}	0.03^{bc}	0.02^{c}	0.003	***	***	**
C12:0	6.18^{a}	3.85^{b}	2.96^{bc}	2.19^{c}	0.166	***	***	ns
iso C13:0	0.06^{a}	0.03^{b}	0.02^{b}	0.02^{b}	0.002	***	***	**
anteiso C13:0	0.01	0.01	0.01	0.01	0.001	ns	**	ns
C13:0	0.10^{a}	0.07^{b}	0.06^{bc}	0.04^{c}	0.003	***	***	*
iso C14:0	0.10	0.11	0.10	0.09	0.003	ns	ns	*
C14:0	13.35^{a}	10.80^{b}	9.69^{bc}	8.46^{c}	0.218	***	***	ns
C14:1 *cis*-9	0.33^{a}	0.22^{b}	0.20^{b}	0.16^{b}	0.009	**	***	**
iso C15:0	0.20^{ab}	0.19^{ab}	0.21^{a}	0.16^{b}	0.004	*	ns	ns
anteiso C15:0	0.49^{a}	0.44^{ab}	0.45^{ab}	0.38^{b}	0.008	*	ns	**
C15:0	1.26^{a}	1.03^{b}	1.04^{b}	0.89^{b}	0.018	***	**	**
isoC16:0	0.29^{a}	0.24^{ab}	0.25^{ab}	0.21^{b}	0.005	**	ns	*
C16:0	29.97^{a}	23.83^{b}	22.45^{bc}	20.96^{c}	0.393	***	***	ns
C16:1 *trans*-6 + *trans*-7	0.06	0.06	0.07	0.07	0.001	ns	ns	**
C16:1 *trans*-8	0.02^{b}	0.04^{ab}	0.03^{ab}	0.07^{a}	0.004	*	ns	**
C16:1 *trans*-9	0.08^{c}	0.25^{bc}	0.33^{ab}	0.56^{a}	0.024	***	**	ns
C16:1 *trans*-10	0.01^{c}	0.01^{b}	0.01^{b}	0.02^{a}	0.001	***	*	ns
C16:1 *cis*-7	0.28	0.27	0.31	0.29	0.005	ns	***	*
C16:1 *cis*-9	1.18^{a}	0.77^{b}	0.73^{b}	0.63^{b}	0.031	*	***	***
C16:1 *cis*-10	0.01^{c}	0.03^{b}	0.02^{bc}	0.05^{a}	0.002	***	***	**
iso C17:0	0.36^{a}	0.32^{b}	0.36^{a}	0.28^{c}	0.005	***	ns	ns
anteiso C17:0	0.50^{a}	0.39^{bc}	0.43^{ab}	0.32^{c}	0.008	***	*	*
C17:0	0.65^{a}	0.52^{bc}	0.60^{b}	0.48^{c}	0.009	***	***	**
C17:1 *cis*-9	0.25^{a}	0.15^{bc}	0.17^{b}	0.11^{c}	0.006	***	***	ns
C18:0 (SA)	5.43^{b}	8.66^{a}	9.82^{a}	9.95^{a}	0.244	***	***	*
C18:1 *trans*-4	0.03^{b}	0.04^{ab}	0.05^{a}	0.04^{ab}	0.002	*	***	ns
C18:1 *trans*-6 + *trans*-8	0.20^{c}	0.46^{b}	0.59^{ab}	0.73^{a}	0.024	***	***	ns
C18:1 *trans*-9	0.22^{c}	0.48^{b}	0.55^{b}	0.70^{a}	0.021	***	**	ns
C18:1 *trans*-10	0.52^{b}	0.99^{ab}	0.85^{ab}	1.80^{a}	0.092	*	ns	ns
C18:1 *trans*-11 (VA)	1.03^{c}	2.99^{bc}	4.06^{ab}	6.20^{a}	0.253	***	*	ns

C18:1 *cis*-9 + t13 + t14	13.29^{b}	17.76^{a}	19.51^{a}	19.19^{a}	0.339	***	***	ns
C18:1 *cis*-10 + t15	0.39^{b}	0.45^{ab}	0.67^{a}	0.69^{a}	0.052	**	***	***
C18:1 *cis*-11	0.42^{b}	0.59^{b}	0.81^{a}	0.79^{a}	0.021	***	***	***
C18:1 *cis*-12	0.28^{d}	0.85^{b}	0.61^{c}	1.26^{a}	0.041	***	***	ns
C18:1 *cis*-13	0.02^{b}	0.04^{b}	0.07^{a}	0.09^{a}	0.003	***	ns	ns
C18:1 *cis*-14 + t16	0.16^{c}	0.21^{c}	0.42^{a}	0.34^{b}	0.013	***	***	ns
C18:2 *trans*-9,*trans*-12	0.42^{c}	0.77^{b}	1.24^{a}	1.24^{a}	0.039	***	ns	ns
C18:1 *cis*-15	0.06^{c}	0.08^{c}	0.24^{a}	0.19^{b}	0.009	***	ns	ns
C18:2 *trans*-8,*cis*13	0.02^{b}	0.03^{b}	0.07^{a}	0.07^{a}	0.003	***	*	ns
C18:2 *cis*-9,*trans*-12	0.08^{c}	0.17^{b}	0.27^{a}	0.25^{a}	0.008	***	ns	ns
C18:2 *trans*-9,*cis*-12	0.15^{c}	0.22^{ab}	0.19^{b}	0.24^{a}	0.004	***	*	ns
C18:2 *n*-6 (LA)	2.66^{b}	4.62^{a}	2.95^{b}	4.82^{a}	0.119	***	***	ns
C18:3 *n*-6	0.10^{a}	0.06^{b}	0.02^{c}	0.03^{c}	0.003	***	*	ns
C18:3 *n*-3 (LNA)	0.74^{c}	0.57^{c}	1.87^{a}	1.42^{b}	0.057	***	ns	***
CLA *cis*-9,*trans*-11 (RA)	0.69^{c}	1.73^{bc}	2.16^{ab}	2.99^{a}	0.116	***	*	ns
C18:4 *n*-3	0.06^{a}	0.04^{b}	0.05^{ab}	0.06^{ab}	0.002	*	***	ns
CLA *trans*-9,*cis*-11+C20:0	0.18^{b}	0.19^{ab}	0.22^{a}	0.21^{ab}	0.004	*	***	**
CLA *trans*-10,*cis*-12	0.01^{b}	0.02^{b}	0.11^{a}	0.09^{a}	0.006	***	***	***
CLA *trans*-11,*cis*-13	0.01^{c}	0.02^{c}	0.16^{a}	0.13^{b}	0.007	***	**	***
CLA *cis*-11,*cis*-13	0.04^{b}	0.04^{b}	0.10^{a}	0.09^{a}	0.003	***	***	***
CLA *trans*-11,*trans*-13	0.08^{c}	0.10^{bc}	0.12^{ab}	0.14^{a}	0.003	***	***	ns
CLA t9,t11 + C20:1 n-9	0.01	0.01	0.01	0.01	0.001	ns	***	*
C20:2 *n*-6	0.02	0.02	0.02	0.02	0.001	ns	**	ns
C20:3 *n*-9	0.06^{a}	0.04^{b}	0.06^{a}	0.04^{b}	0.001	***	***	ns
C20:3 *n*-6	0.03^{ab}	0.03^{a}	0.02^{c}	0.02^{bc}	0.001	***	***	**
C20:4 *n*-6	0.15^{a}	0.15^{a}	0.07^{b}	0.07^{b}	0.004	***	***	***
C20:3 *n*-3	0.01^{bc}	0.01^{c}	0.02^{a}	0.01^{b}	0.001	***	***	ns
C22:0	0.09^{ab}	0.07^{c}	0.11^{a}	0.08^{bc}	0.003	***	***	**
C20:4 *n*-3	0.02^{ab}	0.01^{b}	0.02^{a}	0.02^{ab}	0.001	**	***	**
C22:1 *n*-11	0.00^{b}	0.00^{b}	0.01^{a}	0.01^{ab}	0.001	*	ns	ns
C20:5 *n*-3 (EPA)	0.07^{a}	0.03^{c}	0.07^{a}	0.05^{b}	0.002	***	***	*
C22:2 *n*-6	0.04^{a}	0.03^{b}	0.05^{a}	0.03^{b}	0.001	***	**	**
C22:4 *n*-6	0.01^{ab}	0.01^{a}	0.00^{b}	0.00^{b}	0.001	**	***	***
C24:0	0.02^{a}	0.01^{b}	0.02^{a}	0.02^{ab}	0.001	**	ns	***
C22:5 *n*-3 (DPA)	0.07^{a}	0.04^{b}	0.08^{a}	0.05^{b}	0.002	***	***	***
C22:6 *n*-3 (DHA)	0.02	0.01	0.02	0.02	0.001	ns	ns	ns
Groups of FA								
SFA	75.07^{a}	63.66^{b}	59.17^{b}	53.17^{c}	0.921	***	***	ns
UFA	24.93^{c}	36.34^{b}	40.83^{b}	46.83^{a}	0.921	***	***	ns
MUFA	19.20^{c}	27.36^{b}	30.85^{b}	34.71^{a}	0.652	***	***	ns

PUFA	5.73^{c}	8.98^{b}	9.98^{ab}	12.12^{a}	0.283	***	***	ns
UFA:SFA	0.33^{c}	0.58^{b}	0.70^{b}	0.90^{a}	0.025	***	***	*
PUFA:SFA	0.08^{c}	0.14^{b}	0.17^{b}	0.23^{a}	0.007	***	***	ns
TFA	2.99^{c}	7.07^{b}	9.07^{ab}	12.93^{a}	0.462	***	***	ns
BCFA	2.01^{a}	1.73^{bc}	1.84^{ab}	1.46^{c}	0.029	***	ns	*
OBCFA	3.88^{a}	3.19^{b}	3.35^{b}	2.70^{c}	0.054	***	ns	**
SCFA	16.26^{a}	13.30^{b}	10.73^{bc}	8.73^{c}	0.354	***	***	*
MCFA	55.84^{a}	43.68^{b}	40.56^{b}	36.47^{c}	0.795	***	***	ns
LCFA	27.90^{d}	43.01^{c}	48.71^{b}	54.80^{a}	1.110	***	***	*
PUFA *n*-3	0.98^{c}	0.72^{c}	2.14^{a}	1.63^{b}	0.060	***	*	***
PUFA *n*-6	3.00^{b}	4.92^{a}	3.13^{b}	4.99^{a}	0.118	***	***	ns
n-6:*n*-3	3.12^{b}	7.01^{a}	1.47^{c}	3.09^{b}	0.227	***	***	***
Total CLA	1.02^{c}	2.12^{bc}	2.88^{ab}	3.66^{a}	0.132	***	***	ns
Total C18:1	16.62^{c}	25.28^{b}	28.76^{b}	32.62^{a}	0.667	***	***	ns
Total C18:2	4.35^{c}	7.92^{b}	7.60^{b}	10.28^{a}	0.260	***	***	ns
Δ^{9}-desaturase indices								
C10 index	3.75	3.73	3.78	3.26	0.060	ns	**	ns
C14 index	2.38	2.03	200	1.82	0.050	ns	*	**
C16 index	3.73	3.13	3.14	2.91	0.071	ns	*	***
C18 index	71.25^{a}	67.27^{ab}	66.65^{ab}	65.93^{b}	0.382	*	*	*
CLA *cis*-9,*trans*-11 index	40.34^{a}	37.51^{ab}	34.79^{b}	33.20^{b}	0.444	**	ns	ns
Total index	21.23^{b}	28.26^{a}	31.01^{a}	32.08^{a}	0.517	***	***	ns

[a-d]Means within a row with different superscripts are different ($P < 0.05$).

[1]Diet: CON = control diet; GS = diet containing 300 g/d per head of grape seed; LIN = diet containing 220 g/d per head of linseed; MIX = diet containing 300 g/d of grape seed and 220 g/d of linseed per head.

[2]P-value: D = effect of diet; S = effect of sampling date; D × S = effect of diet and sampling date interaction; ns indicates $P > 0.05$; *$P < 0.05$; ** $P < 0.01$; ***$P < 0.001$.

[3]FAME = fatty acid methyl esters; SA = stearic acid; VA = vaccenic acid; LA = linoleic acid; LNA = linolenic acid; RA = rumenic acid; EPA = eicosapentaenoic acid; DPA = docosapentaenoic acid; DHA = docosahexaenoic acid; SFA = saturated fatty acids, sum of the individual saturated fatty acids reported in this table; UFA = unsaturated fatty acids, sum of the individual unsaturated fatty acids reported in this table; MUFA = monounsaturated fatty acids, sum of the individual monounsaturated fatty acids reported in this table; PUFA = polyunsaturated fatty acids, sum of the individual polyunsaturated fatty acids reported in this table; TFA = *trans* fatty acids, sum of the individuals *trans* fatty acids reported in this table (except CLA isomers); BCFA = branched-chain fatty acids, sum of iso- and anteiso-FA reported in this table; OBCFA = odd- and branched-chain fatty acids, sum of odd-, iso- and anteiso-FA reported in this table; SCFA = short-chain fatty acids, sum of the individual fatty acids from C4:0 to C10:0 reported in this table; MCFA = medium-chain fatty acids, sum of the individual fatty acids from C11:0 to C17:0 reported in this table; LCFA = long-chain fatty acids, sum of the individual fatty acids from C18:0 to DHA reported in this table; PUFA *n*-3 = sum of individual *n*-3 fatty acids reported in this table; PUFA *n*-6 = sum of individual *n*-6 fatty acids reported in this table; CLA = sum of individual conjugated of linoleic acids reported in this table.

543 **Table 3.** Eigenvectors and eigenvalues of correlation matrix based on groups of milk fatty acids,
544 sorted by decreasing values of the PC1

Item[1]	PC1	PC2
UFA	0.246	–0.027
MUFA	0.244	–0.058
Total C18:1	0.243	–0.058
LCFA	0.243	–0.055
PUFA	0.241	0.044
Total C18:2	0.235	0.154
TFA	0.233	0.055
Total CLA	0.231	–0.004
h:H	0.230	–0.099
Total C18:1-*trans*	0.227	0.085
Total C18:1-*cis*	0.195	–0.188
PUFA *n*-6	0.158	0.455
PUFA *n*-3	0.139	–0.470
n-6:*n*-3	–0.021	0.559
BCFA	–0.174	–0.288
OBCFA	–0.202	–0.249
SCFA	–0.231	0.108
TI	–0.236	0.093
MCFA	–0.238	0.030
AI	–0.238	0.021
SFA	–0.246	0.027
Eigenvalues	16.45	2.51
% variance explained	78.3	11.9

545 [1]Item: UFA = unsaturated fatty acids, sum of the individual unsaturated fatty acids reported in table
546 2; MUFA = monounsaturated fatty acids, sum of the individual monounsaturated fatty acids reported
547 in Table 2; LCFA = long-chain fatty acids, sum of the individual fatty acids from C18:0 to DHA
548 reported in table 2; PUFA = polyunsaturated fatty acids, sum of the individual polyunsaturated fatty
549 acids reported in Table 2; TFA = *trans* fatty acids, sum of the individuals *trans* fatty acids reported
550 in table 2 (except CLA isomers); Total CLA = sum of individual conjugated of linoleic acids reported
551 in table 2. h:H = hypocholesterolemic to hypercholesterolemic ratio. PUFA *n*-6 = sum of individual
552 *n*-6 fatty acids reported in table 2; PUFA *n*-3 = sum of individual *n*-3 fatty acids reported in table 2;
553 BCFA = branched-chain fatty acids reported in table 2; OBCFA = odd- and branched-chain fatty
554 acids reported in Table 2; SCFA = short-chain fatty acids, sum of the individual fatty acids from C4:0
555 to C10:0 reported in table 2; TI = trombogenic index; MCFA = medium-chain fatty acids, sum of the
556 individual fatty acids from C11:0 to C17:0 reported in table 2; AI = Atherogenic index; SFA =
557 saturated fatty acids, sum of the individual saturated fatty acids reported in table 2.
558

559 **Tables 4.** Dietary effects on PC scores of individuals belonging to the different dietary treatments for
560 PC1 (*PUFA intake*) and PC2 (*n-6:n-3*)

	Diets[1]				SEM	*P*-value
Item	CON	GS	LIN	MIX		Diet
PC1	–5.7720[d]	–0.2671[c]	1.5283[b]	4.5108[a]	0.5905	< 0.0001
PC2	–0.1499[b]	1.8599[a]	–2.0293[c]	0.3193[b]	0.3105	< 0.0001

561 [a-d]Means within a row with different superscripts are different ($P < 0.05$).
562 [1]Diet: CON = control diet; GS = diet containing 300 g/d per head of grape seed; LIN = diet containing
563 220 g/d per head of linseed; MIX = diet containing 300 g/d of grape seed and 220 g/d of linseed per
564 head.
565

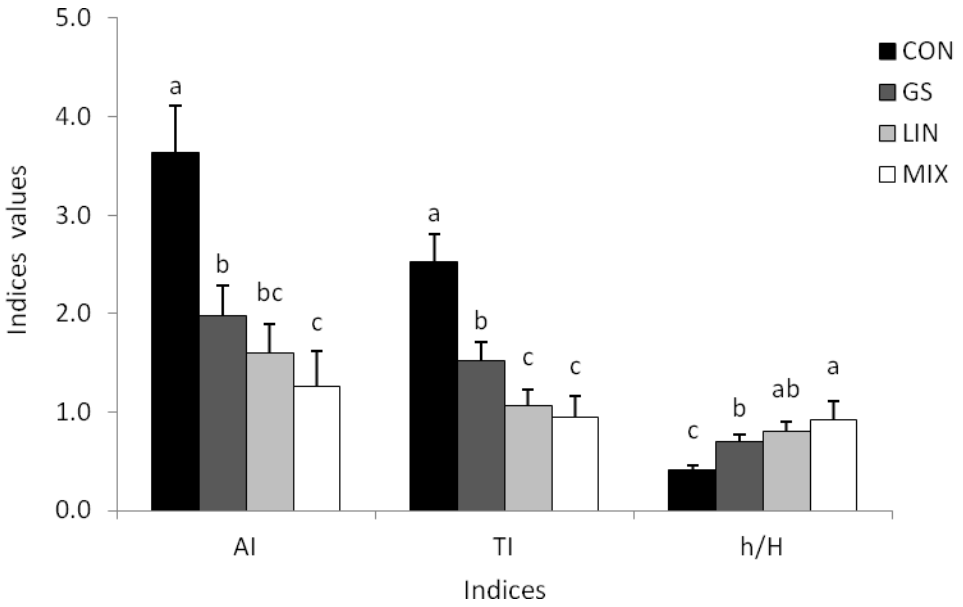
Figure 1. Effect of experimental diets on milk fat nutritional indices: atherogenic index (AI), thrombogenic index (TI) and hypocholesterolemic to hypercholesterolemic ratio (h:H). CON: control diet, GS: diet containing grape seed, LIN: diet containing linseed, MIX: diet containing both grape seed and linseed.

Figure 2. Plot of the scores of the first two principal components of individuals belonging to the different experimental diets. CON: control diet, GS: diet containing grape seed, LIN: diet containing linseed, MIX: diet containing both grape seed and linseed.

Figure 3. Hierarchical cluster analysis results for milk of the four dietary treatments. CON: control diet, GS: diet containing grape seed, LIN: diet containing linseed, MIX: diet containing both grape seed and linseed. (Data from groups of FA + nutritional indices).

577 Correddu, **Figure 1.**

578

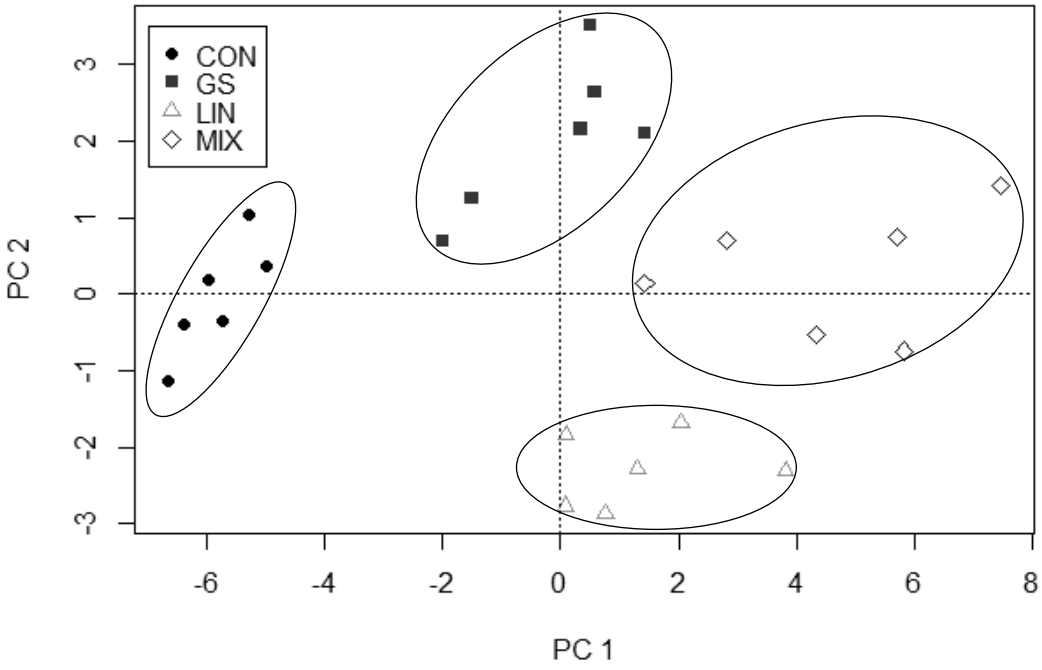


579

580

581 Correddu, **Figure 2.**

582

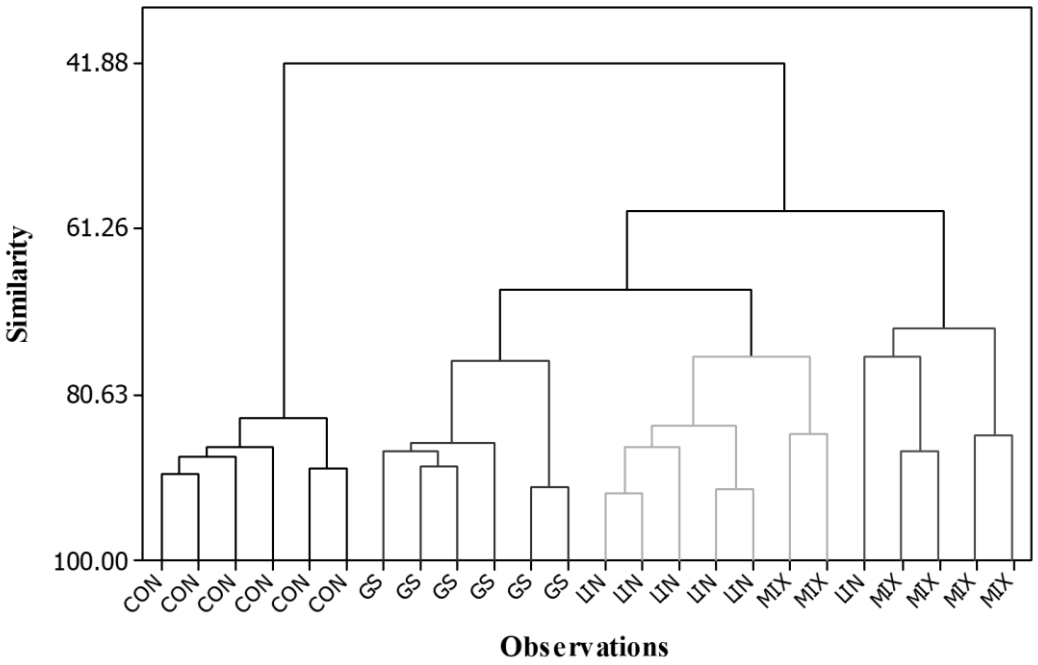


583

584

585 Correddu, **Figure 3.**

586



587